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(54) Gonadotropin releasing hormone analogues

(57) Gonadotropin releasing hormone analogues of formula:

Pyroglu-His-Trp-Ser-A-B-C-D-Pro-E

wherein A is His or Tyr;

B is D-Arg, D-Lys, D-Trp, D-Ala, D-Nal (2) or D-(tBu) Ser;

C is Leu or Trp;

D is Arg, Gln, Tyr or Leu;

E is Gly-NH₂, D-Ala-NH₂, NEt or azaGly provided that, when A is His, C is Leu, D is Arg and E is Gly-NH₂, then B is other than D-Tyr.

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ANALOGUES OF GONADOTROPIN RELEASING HORMONE.

This invention relates to novel peptides and to their uses.

Gonadotropin releasing hormone (GnRH) is a decapeptide which is secreted by the hypothalamic region of the brain. Mammalian gonadotropin releasing hormone has the following amino acid sequence p Glu- His- Trp- Ser- Tyr- Gly- Leu- Arg- Pro-Gly NH₂. There are however variant forms of mammalian GnRH in other vertebrates, for example fish and birds.

The following is a table in which the primary structures of GnRHs isolated from the brains of different vertebrates is depicted.

Mammal	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH ₂
Chicken I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH ₂
Salmon	pGlu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH ₂
Chicken II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH ₂

GnRH and its analogues stimulate the release of pituitary gonadotropin hormones and these in turn activate gametogenesis and steroidogenesis in the gonads. Increased release of gonadotropin hormones has been shown to stimulate the reproductive process in animals and hence methods of increasing the release of gonadotropin hormones has application in the medical and agricultural spheres.

GnRH is also present in, and has effects on, the placenta, central nervous system, adrenals, gonads, and mammary tissue in mammals and hence GnRH analogues also have potential in medical and veterinary applications in regulating these tissues.

It is thus desirable that further and more effective analogues of GnRH be found to increase or decrease the release of gonadotropin hormones in various different classes of vertebrates.

Attempts have also been made to increase the gonadotropin releasing activities of the various primary vertebrate GnRHs in mammals by incorporating D-amino acids into their structure.

In an article in Endocrinology, volume 124, pages 1830 to 1840, 1989 by J A King et al there is disclosed a chimaeric analogue of a naturally occurring vertebrate gonadotropin releasing hormone, pGlu-His-Trp-Ser-His-D-Tyr-Leu-Arg-Pro-Gly.NH₂.

According to one aspect of the invention there is provided a peptide which is a chimaeric analogue of a naturally occurring vertebrate gonadotropin releasing hormone of the formula;

pGlu-His-Trp-Ser-A-B-C-D-Pro-E

wherein:

A is His or Tyr;

B is D-Arg, D-Lys, D-Trp, D-Ala, D-Tyr, D-Nal(2), or D-tert-but-Ser,

C is Leu or Trp;

D is Arg, Gln, Tyr, or Leu; and

E is Gly.NH₂, D-Ala.NH₂, NEt or aza-Gly,

with the proviso that when A is His, C is Leu, D is Arg and E is Gly.NH₂ then B is not D Tyr.

By "chimaeric analogue" is meant a vertebrate gonadotropin releasing hormone wherein one or more of the amino acids at positions 5,7 or 8 are substituted with a different amino acid occurring in the corresponding position in another naturally occurring vertebrate gonadotropin releasing hormone.

Preferred peptides according to the invention include:

pGlu - His - Trp - Ser - His - D - Trp - Leu - Arg - Pro - Gly NH₂

I

pGlu - His - Trp - Ser - Tyr - D - Trp - Trp - Arg - Pro - Gly NH₂

II

pGlu - His - Trp - Ser - His - D - Trp - Trp - Arg - Pro - Gly NH₂

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III

pGlu - His - Trp - Ser - Tyr - D - Trp - Trp - Tyr - Pro -
Gly NH₂

IV

pGlu - His - Trp - Ser - Tyr - D - Arg - Trp - Tyr - Pro -
Gly NH₂

V

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Tyr - Pro -
Gly NH₂

VI

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Gln - Pro -
Gly NH₂

VII

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Leu - Pro -
Gly NH₂

VII

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Arg - Pro -
Gly NH₂

IX

pGlu - His - Trp - Ser - His - D - Arg - Trp - Arg - Pro -
Gly NH₂

X

In another aspect of the invention a peptide which is an analogue of a vertebrate gonadotropin releasing hormone having one of the following formulae is provided:

pGlu - His - Trp - Ser - Tyr - D - Ala - Leu - Gln - Pro -
Gly NH₂

XIII

pGlu - His - Trp - Ser - Tyr - D - Trp - Leu - Gln - Pro -
NEt

XIV

pGlu - His - Trp - Ser - Tyr - D - Arg - Trp - Leu - Pro -
Gly NH₂

XV

pGlu - His - Trp - Ser - His - D - Trp - Trp - Tyr - Pro -
Gly - NH₂

XVI

pGlu - His - Trp - Ser - His - D - Lys - Trp - Tyr - Pro -
Gly NH₂

XVII

The peptides of the invention have agonist properties relative to a naturally occurring vertebrate gonadotropin releasing hormone.

In yet another aspect of the invention there is provided the use of a peptide described above as a regulator for regulating the release of hormones in vertebrates and in particular the use of a peptide of any one the formulae IV, VI, VIII, IX, XV, XVI or XVII to regulate the release of hormones in chickens.

Peptides of the invention are decapeptides having a sequence of ten amino acids. The first amino acid in the sequence occupies position one, the second amino acid in the sequence occupies position two and similarly for amino acids 3 to 10.

The invention provides a new set of GnRH analogues with altered activity compared with native mammalian GnRH of both mammalian and non-mammalian species. Some of the analogues of the invention are chimaeric GnRH analogues in which the amino acid at one of the variable positions along the ten amino acid sequence, namely positions 5, 7 or 8, is substituted with a different amino acid which occurs in the same position in another vertebrate GnRH. The chimaeric analogues of the invention further incorporate a D-amino acid at position 6 in their amino acid sequence. The chimaeric analogues may also be substituted at the carboxyl terminus with N-ethylamide (NEt) or D-Ala.NH₂ or aza-Gly to enhance their activity. These are represented in Table 1.

The invention also provides a number of novel analogues of naturally occurring gonadotropin releasing hormones which include a D-amino acid in their amino acid sequence. One particular analogue of the invention has a further substitution in that Gly. NH₂, which occupies position 10 along the amino acid sequence, is substituted with N-ethylamide (NEt). These are represented in Table 2.

To delineate the functional importance of the variant amino acids in positions 5, 7 and 8, chimaeric analogues according to the invention were tested for luteinizing hormone (LH) and

follicle stimulating hormone (FSH) releasing activities and for receptor binding activity in rat, sheep and chicken pituitaries.

The present study has investigated the activities of GnRH analogues incorporating combinations of the substitutions: His⁵, Trp⁷, Leu⁸, Gln⁸ and Tyr⁸ in mammalian GnRH together with the incorporation of D-Trp⁶, D-Lys⁶ and D-Arg⁶ on receptor binding and gonadotropin hormone release in sheep, rat and chicken pituitaries.

In the following examples the structure of each peptide is indicated by showing any substitution that it has in its amino acid sequence relative to the amino acid sequence of native mammalian gonadotropin releasing hormone. The position, in the amino acid sequence, of the substitution is indicated by a number in superscript. If no substitution is indicated in any particular position, such position is occupied by the amino acid, present in that position, in native mammalian gonadotropin releasing hormone. Therefore, for example, [His⁵ - D - Trp⁶]GnRH represents an analogue of mammalian gonadotropin releasing hormone with the amino acid Histidine in position 5 and the amino acid D - Tryptophan in position 6.

The peptides were prepared with an automated program on a Beckman System 990 peptide synthesizer. Protected peptides

were assembled by standard solid-phase methodology on p-methylbenzhydrylamine-1% divinylbenzene-styrene copolymer resin (3, 4) utilising 50% trifluoroacetic acid in dichloromethane for N-terminal deprotection and dicyclohexylcarbodiimide for the coupling of the following Boc-protected amino acids : Gly, Pro, Arg(Tos), Gln(Xan), Tyr(Cl2-Bzl), Lys(Cl-z), Leu.H2O, Trp, D-Trp, Glu(OBzl), His(Tos) and Ser(Bzl) as well as z-pGlu.

Peptides were cleaved from the resin and deprotected using 1% anisole in redistilled hydrogen fluoride (5). The peptide-ethylamide was cleaved with redistilled ethylamine and deprotected with hydrogen fluoride. The peptides were purified to greater than 96% homogeneity (based on the ratio of the integrated area under the main peak vs. the total integrated area recorded at 210 nm) by preparative C18 reversed-phase high performance liquid chromatography using a 0.25M triethylammonium phosphate pH 2.25/acetonitrile buffer system with desalting in a 0.1% trifluoroacetic acid/acetonitrile. Structures of peptides were confirmed by routine amino acid analysis.

Cultured chicken pituitary cell bioassay

Anterior pituitaries were removed from chicken heads (Golden Grove Poultry Co., Cape Town) which had been kept on ice and washed with disinfectant ('Hibitane'; 0.5% chlorhexidine gluconate in 80% methanol), within 2 h of death. The pituitaries were collected into Dulbecco's Modified Eagles Medium (Gibco, Grand Island, NY) buffered with 20 mM HEPES (pH 7.4) at room temperature, minced with a razor blade, and digested with collagenase for 1 h at 37°C while undergoing

continuous agitation by a slowly rotating stirrer. The collagenase solution consisted of 0.9% collagenase (w/v) (155 U/mg, Worthington Biochemical Corp., Freehold, NJ) and 18mg/1 deoxyribonuclease (Miles Laboratories, Elkhart, IN) in HEPES-BSA buffer containing (mM): NaCl 137, KCl 5, Na₂HPO₄ 0.7, HEPES 25 (pH 7.2), CaCl₂ 0.36, glucose 10 and 1% (w/v) BAS (fatty-acid free, Pentex fraction V, Miles Laboratories). At 10-min intervals the cells were triturated with a 5 ml pipette. The cell suspension was centrifuged twice at 500g for 5 min and the pellet resuspended each time in buffer A [NaCl 140mM, KCl 4mM, Na₂HPO₄ 1.4 mM, glucose 8.3 mM, HEPES 20 mM (pH 7.4) and phenol red 6 mg/1] to which 0.5 mM EDTA and 0.3% (w/v) BSA was added. The cell suspension was then filtered through nylon gauze prior to dilution into Dulbecco's Modified Eagles Medium with 10% foetal calf serum (Gibco), penicillin (60 mg/1), streptomycin (100 mg/1), neomycin (100 mg/1) and amphotericin B (20 mg/1) and dispensed into plastic tissue culture wells (Falcon, Oxnard, CA) at a density of 1,3 pituitary equivalents per well (6-well plates). One pituitary equivalent represents 5.0 to 6.7 x 10⁵ cells. The cells were cultured at 37°C in 5% CO₂ for 24 h, after which the medium was replaced with fresh amphotericin B-free medium. Culture was then continued for a further 24 h.

Prior to stimulation, the cells were washed twice with buffer A containing 1 mM CaCl₂ and 0.1% BSA and pre-incubated twice for 5 min in the same buffer. Stimulations were carried out for 30 min at 37°C in buffer A with 1 mM CaCl₂ and 0.1% BSA. The medium was collected, centrifuged and stored at -20°C until radioimmunoassay of chicken LH. Mammalian GnRH was included in all bioassays as a reference standard for the comparative activity of the other GnRH analogues.

Cultured sheep pituitary cell bioassay

The biological activity of the synthetic GnRHs was assessed using cultured sheep anterior pituitary cells as previously described (1, 2). Anterior pituitaries were dissected from sheep within 30 min of slaughter, diced and incubated in collagenase solution as described for chicken pituitaries. The cells were resuspended in Minimum Essential Medium - Eagle (MEM) (with Hank's salts) (Gibco, Paisley, Scotland) containing 10% foetal calf serum (Gibco) and antibiotics and cultured (10^5 - 10^5 /dish) at 37°C in 5% CO₂ for 4 or 5 days, with the medium being replaced after 3 days.

Prior to stimulation the cells were washed twice with MEM containing 10 % foetal calf serum and 4 times with serum-free MEM. Peptide stimulations (in triplicate) were conducted over 2 h in 1 ml serum-free MEM at 37°C in 5% CO₂. 900 μ l of medium from each test was collected into tubes containing 100 μ l of HEPES-BSA buffer, centrifuged and stored at -20°C until radioimmunoassay. Mammalian GnRH was included in all bioassays as a reference standard for the comparative activity of the other GnRH analogues.

Gonadotropin radioimmunoassays

Ovine LH radioimmunoassays (RIA) were performed with duplicate aliquots of conditioned medium (or dilutions thereof) from stimulation experiments as previously described (6), except that the second antibody was linked to cellulose (Sac-Cel RD70, Wellcome Reagents Limited, Beckenham, England). Purified ovine LH (LER- 1056-C2, gift from L.E. Reichert) was iodinated by the chloramine-T method and free

iodide was removed on a cellulose CF 11 (Whatman Inc., Clifton, NJ) column. A standard curve was generated with unlabelled sheep LH (NIH-LH-S18) and antiserum GDN 15 (gift from G.D. Niswender, Colorado State University) which had been raised against sheep LH.

Chicken LH was assayed as previously described (7), except that the second antibody was linked to cellulose (Sac-Cel RD70).

Ovine FSH radioimmunoassays employed anti-oFSH-1 antiserum (final dilution 1:160,000), radioiodinated oFSH-I-1 and oFSH-RP-1 as standard. All reagents were supplied by S. Raiti (National Hormone and Pituitary Program, NIDDK) and utilised as instructed. Separation of bound and free ^{125}I -oFSH was achieved with a second antibody conjugated to cellulose (Sac-Cel RD70). All comparative studies between analogues were performed in the same radioimmunoassay and the intra-assay coefficient of variation for radioimmunoassays were : 13.7% (chicken LH), 4.6% (sheep LH), 8.3% (sheep FSH).

Gonadotropin releasing hormone receptor binding assays

The binding of synthetic vertebrate gonadotropin releasing hormones and analogues thereof to gonadotropin releasing hormone receptors in rat anterior pituitary membranes was investigated as previously described (1, 2). In the present study a sheep pituitary gonadotropin releasing hormone receptor binding assay was also developed.

Anterior pituitaries from adult male rats (Long-Evans) or castrated adult male sheep were homogenised in ice-cold 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM dithiothreitol, 1.0

mM EDTA and 0.1% BSA. The rat pituitary homogenate was centrifuged at 15,000 x g for 30 min at 4°C to yield a membrane pellet which was resuspended in the Tris-HCl buffer. The sheep pituitary homogenate, however, was centrifuged at 100 x g (5 min, 4°C) and the supernatant recentrifuged at 3500 x g for 10 min at 4°C. The resulting pellet was resuspended in the Tris-HCl buffer. The binding assay comprised incubation of the membrane preparation (0.16 rat or 0.05 sheep pituitary equivalents/tube), 60,000 cpm ¹²⁵I-[D-Ala⁶-N Me-Leu⁷, Pro⁹-NHet]GnRH [radioiodinated by a modification of the chloramine-T method (8) - specific activity by the self-displacement method, 1000-1600 Ci/ g] and increasing concentrations of the test peptides for 90 min at 4°C. The assay was performed in glass test-tubes (12 x 75 mm) with a total volume of 0.5 ml 10 mM Tris-HCl buffer pH 7.4 containing 1 mM dithiothreitol, 1 mM EDTA and 0.1% BSA. The incubation was terminated by dilution with 3 ml ice-cold phosphate buffered saline, pH 7.4, containing 1% BSA (PBS-BSA), followed by immediate vacuum filtration through glass-fibre filters (GF/C; Whatman) presoaked in PBS-BSA. The filters were washed twice with 3 ml PBS-BSA, and the retained radioactivity counted. Non-specific binding was determined in the presence of 10⁻⁶ M unlabelled [D-Ala⁶, N Me-Leu⁷, Pro⁹-NHet]GnRH and subtracted from all samples (2% in rat and 26% in sheep).

Data reduction and potency estimates

ED₅₀ values (concentrations required for half maximal gonadotropin release in bioassays or for half maximal inhibition of ¹²⁵I-[D-Ala⁶-N MeLeu⁷, Pro⁹-NHet]GnRH binding) of GnRH analogues were determined using a four-parameter, nonlinear curve fitting program (Allfit), forcing curves to

share basal and maximal values. K_d values were calculated from the ED_{50} s by the method of Cheng and Prusoff (9). Standard errors of the mean were derived from Allfit estimates. Relative potencies were calculated from ED_{50} and K_d values relative to those for mammalian GnRH. All values presented are the means of several independent experiments.

Results

All of the analogues tested had enhanced LH-releasing activity in chicken pituitary cell cultures when compared with mammalian GnRH. Since mammalian GnRH and chicken GnRH I in (the releasing hormone in the chicken hypothalamus) have similar activities, the analogues are also more active than chicken GnRH I. Analogues IV, VI, VIII, IX, XV, XVI, and XVII had relative activities 500% or higher compared with mammalian GnRH and most of these are characterised by having D-amino acid-Trp⁷-Tyr⁸ substitutions. In sheep pituitary cell cultures, analogues I, II, XIII and XIV display enhanced LH-releasing activity.

Receptor binding potency of the GnRH chimaeric analogues in sheep and rat pituitary was in general correlated with, LH-releasing potency in the sheep pituitary, however, two classes of analogues emerged in which the relative magnitude of these activities deviated significantly:

- 1 [His⁵-D-Trp⁶]GnRH exhibited higher receptor binding activity than LH-releasing activity. (Table I)

- 2 The analogues with a Trp⁷-Tyr⁸ substitution characteristically displayed LH-releasing activity which is

higher than their relative receptor binding potencies (Table I).

Certain of the analogues, as indicated in Tables 1 and 2, showed potencies for FSH release which were similar to their potencies for LH release, in the sheep pituitary.

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TABLE 1

The Estimated Potencies of Various Chimaeric Analogues of GnRH with regard to
Luteinizing Hormone (LH) Release and Receptor Binding Activity

<u>Chimaeric GnRH Analogues</u>		<u>LH RELEASE*</u>		<u>RECEPTOR BINDING*</u>	
		(chicken pituitary)	(sheep pituitary)	(sheep)	(rat)
I .	His ⁵ -D-Trp ⁶ GnRH	3.5	4.2 **	30.1	57.4
II.	D-Trp ⁶ -Trp ⁷ GnRH	3.1	7.6 **	0.8	9.3
III.	His ⁵ -D-Trp ⁶ -Trp ⁷ GnRH	3.1	1.5 **		
IV	D-Trp ⁶ -Trp ⁷ -Tyr ⁸ GnRH	8.9	0.5 **	0.01	0.25
V.	D-Arg ⁶ -Trp ⁷ -Tyr ⁸ GnRH	4.0	-	-	
VI.	D-Lys ⁶ -Trp ⁷ -Tyr ⁸ GnRH	10.0	-	0.09	
VII.	D-Lys ⁶ -Trp ⁷ -Gln ⁸ GnRH	2.5	-	0.07	
VIII.	D-Lys ⁶ -Trp ⁷ -Leu ⁸ GnRH	6.7	0.1	0.02	
IX.	D-Lys ⁶ -Trp ⁷ GnRH	5.0	2.0	1.1	
X.	His ⁵ -D-Arg ⁶ -Trp ⁷ GnRH	2.1	1.0	0.8	

*Potency relative to mammalian GnRH

GnRH refers to pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly NH₂

**Similar potencies for FSH release were obtained

TABLE 2

The Estimated Potencies of Various Analogues of GnRH with regard to
Lutenizing Hormone (LH) Release and Receptor Binding Activity

<u>GnRH Analogues</u>	<u>LH RELEASE*</u>		<u>RECEPTOR BINDING*</u>
	(chicken pituitary)	(sheep pituitary)	(sheep): (rat)
<u>CHICKEN GnRH I Analogues</u>			
XIII. D-Ala ⁶ -Gln ⁸ GnRH	2.1	2.6 **	
XIV D-Trp ⁶ -Gln ⁸ -Pro ⁹ NET GnRH	2.0	4.4 **	1.3
<u>SALMON GnRH ANALOGUES</u>			
XV D-Arg ⁶ -Trp ⁷ -Leu ⁸ GnRH	5.4		0.09
<u>CHICKEN GnRH II ANALOGUES</u>			
XVI His ⁵ -D-Trp ⁶ -Trp ⁷ -Tyr ⁸ GnRH	6.9	0.4 **	0.02
XVII His ⁵ -D-Lys ⁶ -Trp ⁷ -Tyr ⁸ GnRH	5.0	0.5	0.2

*Potency relative to mammalian GnRH

GnRH refers to pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly NH₂

**Similar potencies for FSH release were obtained

CLAIMS:

1. A peptide which is a chimaeric analogue of a naturally occurring vertebrate gonadotropin releasing hormone of the formula

pGlu-His-Trp-Ser-A-B-C-D-Pro-E

wherein

A is His or Tyr;

B is D-Arg, D-Lys, D-Trp, D-Ala, D-Tyr, D-Nal(2) or D-tert-but-Ser;

C is Leu or Trp;

D is Arg, Gln, Tyr or Leu; and

E is Gly.NH₂, D-Ala.NH₂, N^εEt or aza-Gly,

with the proviso that when A is His, C is Leu, D is Arg and E is Gly.NH₂ then B is not D-Tyr.

2. A peptide according to claim 1 which is:

pGlu - His - Trp - Ser - His - D - Trp - Leu - Arg - Pro - Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Trp - Trp - Arg - Pro - Gly NH₂;

pGlu - His - Trp - Ser - His - D - Trp - Trp - Arg - Pro - Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Trp - Trp - Tyr - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Arg - Trp - Tyr - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Tyr - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Gln - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Leu - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Lys - Trp - Arg - Pro -
Gly NH₂; or

pGlu - His - Trp - Ser - His - D - Arg - Trp - Arg - Pro -
Gly NH₂;

3. A peptide which is an analogue of a vertebrate
gonadotropin releasing hormone which is:

pGlu - His - Trp - Ser - Tyr - D - Ala - Leu - Gln - Pro -
Gly NH₂;

pGlu - His - Trp - Ser - Tyr - D - Trp - Leu - Gln - Pro -
NET;

pGlu - His - Trp - Ser - Tyr - D - Arg - Trp - Leu - Pro -

Gly NH₂;

pGlu - His - Trp - Ser - His - D - Trp - Trp - Tyr - Pro -
Gly - NH₂; or

pGlu - His - Trp - Ser - His - D - Lys - Trp - Tyr - Pro -
Gly NH₂

4. A peptide according to claim 1 for use in a method of regulating the release of hormones in vertebrates.
5. A peptide according to claim 2 for use in a method of regulating the release of hormones in vertebrates.
6. A peptide according to claim 3 for use in a method of regulating the release of hormones in vertebrates.
7. A pharmaceutical composition for use in the regulation of the release of hormones in vertebrates, which comprises at least one peptide as claimed in any one of claims 1 to 6.
8. A peptide which is a chimaeric analogue of a naturally occurring vertebrate gonadotropin releasing hormone are substantially as hereinbefore described.
9. A peptide which is a chimaeric analogue of a naturally occurring vertebrate gonadotropin releasing hormone and substantially as hereinbefore described with reference to the examples.